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[COMMUNICATION]

In vitro Spermatogenesis in *Oryctes rhinoceros* (Coleoptera, Scarabaeidae): The Role of Ecdysone and Juvenile Hormone

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ABSTRACT—The hormonal factors which regulate spermatogenesis in *Oryctes rhinoceros* were investigated *in vitro*. Spermatogonial multiplication in cultures occurred in the presence of testis sheath which is the proposed site of ecdysteroid production. Meiosis of spermatocytes occurred when exogenous ecdysone (20-hydroxy ecdysone) was added to the culture. The secondary spermatocytes thus developed underwent spermiogenesis only when active corpus allatum was introduced.

INTRODUCTION

The endocrine control of spermatogenesis in insects differs according to species. Ecdysone stimulates spermatogonial multiplication in *Rhodnius prolixus* [1] and in *Locusta migratoria* [2], and promotes meiosis and spermiogenesis in most of the lepidopteran insects [3–7]. According to Shimizu *et al.* [6], Loeb and Woods [7] and Giebultowicz *et al.* [8] ecdysone is produced in testis. In *Samia cynthia* spermatogenesis continues in the culture medium in the presence of ecdysone and a macromolecular factor [9, 10]. In Diptera, ecdysone apparently has no role during spermatogenesis [11, 12]. Juvenile hormone (JH) and its analogues have in general been found to inhibit spermatogenesis [5, 13, 14]. However, in some insects, topically applied JH analogues promote spermatogenesis [15, 16].

Investigations on the regulatory mechanisms of insect spermatogenesis have been mainly confined to Lepidoptera with relatively little attention being given to other groups. *In vivo* studies of spermatogenesis in the coleopteran insect *Oryctes rhinoceros*, a major pest of the coconut palm, have revealed that even though secondary spermatocytes appear in the pupal stage, spermiogenesis occurs only after adult emergence [17]. It has also been demonstrated that high doses of methoprene topically applied on 0 day-old pupa of this insect inhibit meiosis and spermiogenesis [18]. However, very little is known about the hormonal mechanisms involved in spermatogenesis, and hence the present *in vitro* studies were undertaken in order to elucidate the endocrine regulation of spermatogenesis in this insect.

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MATERIALS AND METHODS

The third instar larvae of *Oryctes rhinoceros* were reared in the laboratory on sterilized cow dung as reported earlier [17]. One hr-old male pupae were sterilized superficially in a mixture of mercuric chloride and ethanol (10 mg $HgCl_2$ dissolved in 50% ethanol) for two minutes. They were washed repeatedly in sterile water and then swabbed in isopropyl alcohol. The testes were dissected under a Laminar flow hood and were placed in Rinaldini's solution [19]. Grace's insect medium and fetal calf serum (GIBCO) compounded in a ratio 100 ml : 20 ml, supplemented with 0.2 ml antibiotic mixture (Penicillin-sodium salt 100,000 units and Streptomycin sulphate 100 mg, each dissolved in 5 ml triple distilled water, along with Gentamycin 1 mg/ml) served as the basic medium (BM) for the present study. 20-Hydroxy

ecdysone (SIGMA) was dissolved in Rinaldini's solution 100 μg/ml, and from this 2×10^{-6}, 5×10^{-6} and 1×10^{-5} molar solutions (M) were prepared to study the dose-effect. The addition of prothoracic gland, with or without the brain, corpora cardiaca and corpora allata complex (BR, CC and CA, respectively) of male pupae also constituted a culture treatment as described in the experimental protocol. The excised testes were teased into smaller pieces after removing the remaining surrounding tissues. Fragments of testis with or without testis sheath were introduced into tissue culture vials with screw caps (12 cm length $\times$1 cm diameter, Borosil) containing 1 ml basic medium (BM). The culture thus prepared was incubated under aseptic conditions at 28°C for 16 to 21 days. The medium was exchanged after 16 days. The percentage of spermatocytes in meiotic and post-meiotic stages were determined in five (50 μl) samples of the medium, after 16 days of incubation, in each of the respective vial (n=8), corresponding to the respective dose. The data were statistically analysed employing Single Factor ANOVAR.

The experimental protocol is as follows:

Group 1. Testis fragments incubated in BM without testis sheath.

Group 2. Testis fragments and BR CC CA of male pupae (n=5) incubated in BM without testis sheath.

Group 3. Testis fragments and 20-hydroxy ecdysone (1×10^{-5} M) incubated in BM without testis sheath.

Group 4. Testis fragments incubated in BM along with testis sheath.

Group 5. Testis fragments, testis sheath and BR CC CA of male pupae (n=5) incubated in BM.

Group 6. Testis fragments, testis sheath, prothoracic gland and BR CC CA of male pupae (n=5) incubated in BM.

Group 7. Testis fragments, testis sheath and 20-hydroxy ecdysone (2×10^{-6} M) incubated in BM.

Group 8. Testis fragments, testis sheath and 20-hydroxy ecdysone (5×10^{-6} M) incubated in BM.

Group 9. Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated in BM.

Group 10. Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days followed by addition of CA of newly emerged adult males (n=5).

Group 11. Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days, followed by addition of BR CC CA of newly emerged adult males (n=5).

Group 12. Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days, followed by addition of CA of one week-old adult males (n=5).

Group 13. Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days, followed by addition of CA of one month-old adult males (n=5).

RESULTS

Results of various culture treatments are presented in Table 1. The testis fragments, when cultured without testis sheath, degenerated after 10 days, although a few spermatogonia had multiplied in the initial stages (Groups 1, 2, 3). On the other hand, testis under culture containing the testis sheath (Group 4) showed abundant spermatogonial multiplication (Fig. 1). However, no further development was observed even with maintenance of the cultures for up to 21 days. The brain, corpora cardiaca, and corpora allata complex of male pupa did not induce any development (Group 5). However, when prothoracic gland was introduced along with the brain complex (Group 6), meiotic stages and a few spermatids appeared. 20-Hydroxy ecdysone accelerated meiosis. In testis cultured with 20-hydroxy ecdysone, primary spermatocytes appeared on the second day and secondary spermatocytes were observed from the 10th day onwards (Groups 7, 8, 9). Furthermore, a dose-dependent increase in meiotic division was observed with the addition of 20-hydroxy ecdysone

TABLE 1. Results of testis culture of 1 hr-old pupae of *O. rhinoceros*

Group	No. of Expts.	Additives to Basic Medium	Incubation period	Results
1	8	Testis fragments only	16	A few spermatogonia; degeneration after 10 days
2	8	〃 〃 and BR CC CA of pupae (n=5)	16	〃 〃 〃
3	8	〃 〃 and 20-hydroxy ecdysone (1×10^{-5} M)	16	〃 〃 〃
4	8	〃 〃 and testis sheath	16	Mitoses of spermatogonia only
5	8	〃 〃 testis sheath and BR CC CA of ♂ pupae (n=5)	16	〃 〃 〃
6	8	〃 〃 testis sheath, prothoracic gland and BR CC CA of ♂ pupae (n=5)	16	A few spermatids and spermatozoa
7	8	〃 〃 testis sheath and 20-hydroxy ecdysone (2×10^{-6} M)	16	Primary spermatocytes on 2nd day, secondary spermatocytes on 10th day cyst degenerated after 21 days
8	8	〃 〃 testis sheath and 20-hydroxy ecdysone (5×10^{-6} M)	16	〃 〃 〃
9	8	〃 〃 testis sheath and 20-hydroxy ecdysone (1×10^{-5} M)	16	〃 〃 〃
10	8	Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days followed by addition of CA of newly emerged ♂ adult (n=5)	10+6	Secondary spermatocytes did not undergo any development
11	8	Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days followed by addition of BR CC CA of newly emerged ♂ adult (n=5)	10+6	Secondary spermatocytes underwent spermiogenesis
12	8	Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days followed by addition of CA of one week old ♂ adult (n=5)	10+6	〃 〃 〃
13	8	Testis fragments, testis sheath and 20-hydroxy ecdysone (1×10^{-5} M) incubated for 10 days followed by addition of CA of one month old ♂ adult (n=5)	10+6	〃 〃 〃

(Fig. 4). The secondary spermatocytes survived up to 21 days without undergoing spermiogenesis, but the cyst began to degenerate (Fig. 2). The corpora allata of newly emerged adult males were introduced into 10 days incubated culture (Group 10); this did not stimulate the development of secondary spermatocytes. However, when the same experimental model was repeated by adding BR CC CA of newly emerged adult males (Group 11), CA of one week-old adult males (Group 12) or CA of one month-old adult males (Group 13) respectively, the secondary spermatocytes underwent spermiogenesis. As a result, spermatids and spermatozoa appeared in the culture vials (Fig. 3).

DISCUSSION

In vivo studies of spermatogenesis in *Oryctes rhinoceros* [17] have shown that spermatogonia occupy the germinal zone of 1 hr-old pupa. Primary spermatocytes normally appear on the 10th hr and meiotic stages are seen in two day-old pupa. However, spermiogenesis is observed only after adult emergence (2–4 days). It has been observed in the present study that in the absence of testis sheath, spermatogonia degenerate. It may be considered that testis sheath secretes factor(s) which sustain spermatogonia in the culture and promote their multiplication. Loeb and Woods [7] and Giebultowicz *et al.* [8] suggested that testis

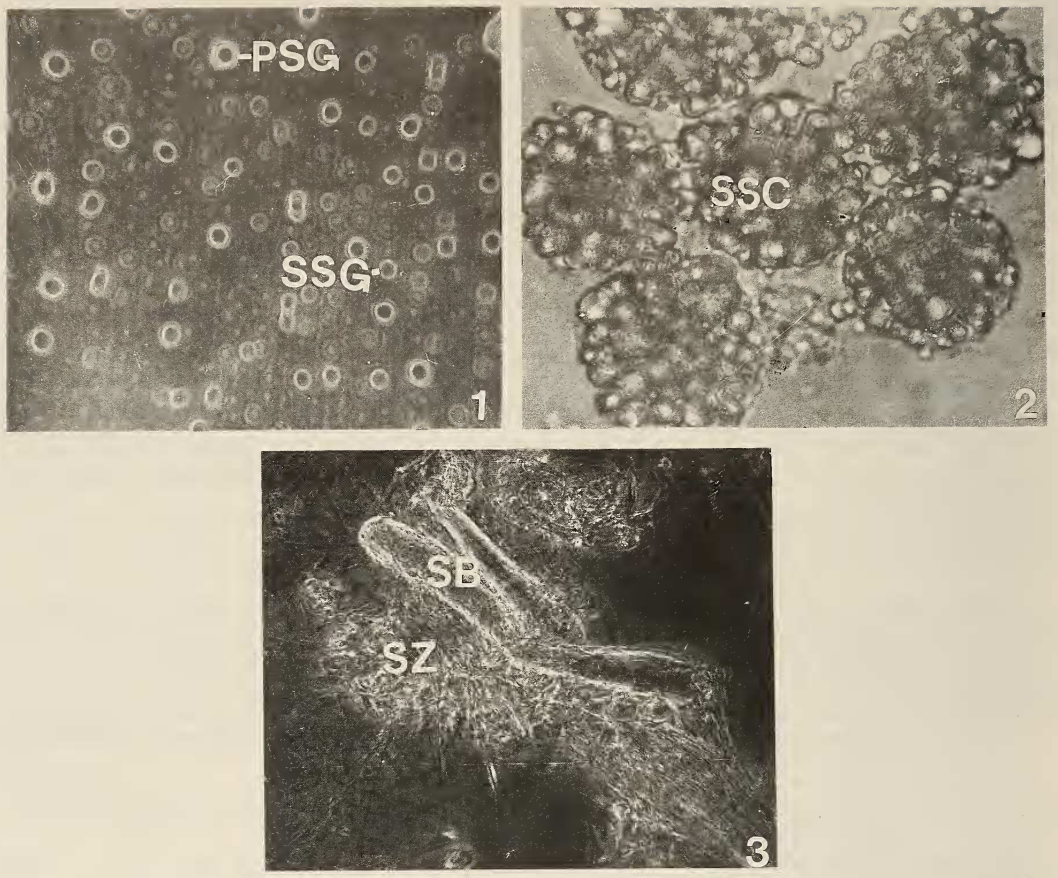

Fig. 1. Spermatogonial multiplication after culturing 1 hr-old pupal testis fragments in BM and testis sheath ×600.
Fig. 2. Secondary spermatocytes in a degenerating condition developed after culturing testis fragments in 20-hydroxy ecdysone and kept beyond 21 days without corpora allata ×600.
Fig. 3. Sperm bundles and released spermatozoa developed after culturing 10 days incubated culture in 20-hydroxy ecdysone, with active corpora allata of 1 month old-adult ×600.
Abbreviations for Figs. 1–3. PSG, primary spermatogonia; SB, sperm bundles; SSC, secondary spermatocytes; SSG, secondary spermatogonia; SZ, spermatozoa.

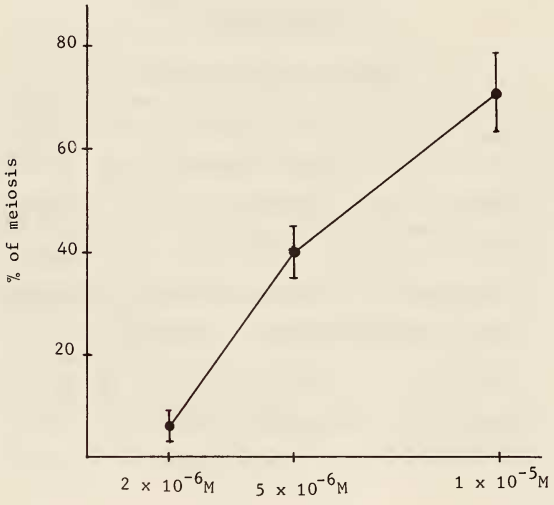

Fig. 4. Showing the effect of three doses of 20-hydroxy ecdysone on the meiotic division of spermatocytes of *O. rhinoceros*. Analysis of variance has shown the dose response to be highly significant ($P<0.05$). I represents SD of meiosis.

sheath produces ecdysteroids.

The present study has revealed that exogenous ecdysone promotes meiosis. In *Rhodnius* [1] and in *Locusta* [2] spermatogonia multiply in the presence of exogenous ecdysone, whereas in Lepidoptera, meiosis and spermiogenesis are promoted by ecdysone [3–7]. In *Oryctes rhinoceros,* the percentage of meiotic and post-meiotic spermatocytes increased with the increase in the dose of 20-hydroxy ecdysone. In the present culture treatments, spermiogenesis occurred only after the introduction of active corpora allata into the medium. It appears that the corpora allata of newly emerged adult males have not attained activity, but brain and corpora cardiaca seem to have induced their activity (Groups 10, 11). In *Oryctes rhinoceros,* the corpus allatum has a stimulatory effect on spermiogenesis. Promotion by juvenile hormone (JH) of any processes related to spermatogenesis as reported here for *Oryctes rhinoceros,* seems unusual. JH inhibits the elongation of spermatids in the testis culture of *Bombyx mori* [5]. JH and its analogues inhibit spermatogenesis in the several other insects [13, 14]. However, JH analogues applied topically have been shown to accelerate spermatogenesis in *Draeculacephala crassicornis* [15] and in *Dysdercus cingulatus* [16]. On the whole it appears that in *Oryctes rhinoceros,* ecdysone as well as juvenile hormone are equally important during spermatogenesis. In the normal insect, spermiogenesis occurs after adult emergence because the corpora allata become active at that time.

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